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ORIGINAL ARTICLE

Growth phase-dependent biosynthesis of Nep, a halolysin-like protease secreted by the alkaliphilic haloarchaeon *Natrialba magadii*

R.A. Paggi, E.A. Madrid, C.P. D'Alessandro, M. Cerletti and R.E. De Castro

Instituto de Investigaciones Biológicas, FCEyN-UNMDP, Funes 3250 4th Floor, Mar del Plata, Argentina

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Correspondence
Rosana E. De Castro, Instituto de Investigaciones Biológicas, Funes 3250 4th Floor, Mar del Plata 7600 CC 1245, Argentina. E-mail: decastro@mdp.edu.ar

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Abstract

Aims: The alkaliphilic haloarchaeon *Natrialba magadii* secretes a halolysin-like protease (Nep) that is active and stable in high salt and in organic solvents, which represents a potential resource for biocatalysis in low water activity conditions. In this study, the effect of the growth stage on Nep biosynthesis was examined.

Methods and Results: Nep mRNA and extracellular protease activity were measured by RT-PCR and azocaseinolytic activity determination, respectively. Increased abundance in Nep mRNA was observed in *Nab. magadii* cells with culture age, which correlated with accumulation of extracellular protease activity. Moreover, a 'stationary phase behavior' on synthesis of Nep was evidenced in low-density cultures incubated with stationary phase medium.

Conclusions: *nep* gene expression is up-regulated during the transition to the stationary phase in response to 'factors' (metabolite and/or regulatory molecule) occurring in high-density cultures of *Nab. magadii*. Although the identity of these molecules remains to be determined, preliminary evidence suggests that they are hydrophobic and stable in high salt and high pH values (3·5 mol l^{-1} NaCl, pH 10).

Significance and impact of study: This study contributes to gain insight into the regulation of haloarchaeal protease biosynthesis, facilitating the large-scale production of this extremozyme for basic studies or potential applications.

Introduction

In addition to the central role that proteases play in cell physiology, proteolytic enzymes (mainly of microbial origin) are extensively used in biotechnology and industry (Rao *et al.* 1998). Haloarchaea secrete extracellular proteases, and a number of these enzymes have been isolated and characterized (De Castro *et al.* 2006; Shi *et al.* 2006; Vidyasagar *et al.* 2006a,b). These extreme halophiles typically require molar salt concentrations (>2 mol l^{-1} NaCl) for cellular integrity and growth. Thus, haloarchaeal enzymes, including proteases, are active and stable in high salt concentrations, and many are also moderately thermophilic and/or alkaliphilic (De Castro *et al.* 2006). These features make haloarchaeal proteases an interesting resource for biocatalysis in low water activity conditions (high salt and/or presence of organic solvents). Studies on the regulation of haloarchaeal protease synthesis are limited (Vidyasagar *et al.* 2006b). Thus, efforts aimed at elucidating the mechanisms and conditions affecting their biosynthesis will help to understand their physiological role and to improve the large-scale production of these extremozymes for basic enzymology and structural studies, as well as for potential applications.

Nep (*Natrialba magadii* extracellular protease) is a halolysin-like serine protease produced by the alkaliphilic haloarchaeon *Nab. magadii* (optimum growth in 3·5 mol l^{-1} NaCl and pH 10). This protease was purified and biochemically characterized (Giménez *et al.* 2000), the gene encoding Nep was cloned, and recombinant

active protease was synthesized in *Haloferax volcanii* (De Castro *et al.* 2008). Nep is active and stable in high salt solutions or in the presence of organic solvents (Ruiz and De Castro 2007), anticipating the potential use of this enzyme in enzyme-catalysed peptide synthesis reactions.

Nep has been detected in the culture medium of *Nab. magadii* cultures upon transition to the stationary phase (Giménez *et al.* 2000), suggesting that it was produced in response to nutrient scarcity, an increase in the population density or both. We have recently shown that accumulation of Nep activity is stimulated by nutrient limitation or slow growth rate, indicating that this protease is induced in response to a deficit in the energetic status of the cells and thus may serve a nutritional purpose (D'Alessandro *et al.* 2007). However, low-density cultures starved for carbon and nitrogen sources did not produce detectable amounts of extracellular protease activity (D'Alessandro *et al.* 2007), which suggested the possibility that *de novo* synthesis of the protease was required and/or 'factors' present in stationary phase cultures may be needed for Nep formation.

In this report, we show that synthesis of Nep is up-regulated as the culture ages in response to 'factors' occurring in high-density cultures of *Nab. magadii*. Attempts were made to identify these 'factors' experimentally and using *in silico* analysis.

Materials and methods

Culture conditions

Nab. magadii ATCC 43099 was grown in Tindall medium containing 20 g l^{-1} of yeast extract Oxoid (Basingstoke, England) at 37°C aerobically (Tindall *et al.* 1984). Cell growth was monitored by the increase in the optical density of the cultures at 600 nm (OD_{600}).

Conditioned media (CM) was prepared from a culture of *Nab. magadii* harvested at OD_{600} ~1·5. Cell-free medium was obtained by centrifugation at 12 000 ***g*** for 20 min followed by ultrafiltration in membranes with a 10 kDa cut-off (Amicon; Millipore, Billerica, MA) to eliminate cells and the extracellular protease present in the medium. A culture of *Nab. magadii* that had been grown exponentially for several doubling times was spliced in two halves at OD_{600} 0·3: one half was centrifuged at 12 000 ***g*** for 20 min and the cells were suspended in one volume of CM supplemented with 10% (v/v) of fresh medium and the other half was used as control (no additions, NA). Both cultures were grown at 37°C aerobically. Samples were withdrawn at OD_{600} representative of exponential (E, OD_{600} 0·5), early stationary (ES, OD_{600} 2·3) and late stationary (LS, OD_{600} 2·7) growth phases for RNA extraction. In addition, samples were taken along the growth curve and centrifuged at 12 000 ***g*** for 10 min. at 4°C to obtain cell-free media for the determination of protease activity.

RNA extraction and RT-PCR

Total RNA was extracted from *Nab. magadii* cells using Trizol reagent Invitrogen (Gaithersburg, MD, USA) according to the manufacture's instructions. RNA was spectrophotometrically quantified, and its quality was assessed on 1% (w/v) agarose/formaldehyde gels. Estimation of Nep transcript abundance was performed by semi-quantitative RT-PCR. RNA samples were treated with RQ1 RNAse-Free DNAse I Promega (Madison, WI, USA), extracted with phenol:chloroform, concentrated by ethanol precipitation and suspended in RNAse-free water containing 5 mmol l^{-1} DTT. The absence of contaminating genomic DNA was assessed by PCR amplification using primers for *Nab. magadii* 16S rRNA. First-strand cDNA was synthesized in a reaction mix (25 μl) containing 200 ng (or 1 μg for 7S RNA) of heat-denatured (5 min, 70°C) RNA, 0·5 mmol l^{-1} of dNTPs mix, 5 mol l^{-1} DTT, 1× RT reaction buffer, 0·5 μg random primer (10 mer, 70% G+C content, 5′-ASSSSSSSSAT-3′) and 200–400 U M-MLV-RT Promega (Madison, USA). The reaction was incubated at 37°C for 1 h and stopped by heating to 70°C for 10 min. PCR amplifications (20 μl) were performed using 1–2 μl first-strand cDNA and 1 μmol l^{-1} primers specific for Nep (Nep-S 5′-ATGACACGTGATACCAATAGTAATGTCG-3′; Nep-AS 5′-AGTTGCTGATGCCGGCGTGTC-3′) and 7S RNA (7S-F 5′-CCAACGTGGAAGCCTCGTC-3′; 7S-R 5′-GGTGGTCCGCTGCTCACTTC-3′). To avoid PCR saturation, different numbers of amplification cycles (15–40) were tested for each cDNA. Finally, cycles of PCR were as follows: Nep (94°C 30 s, 50°C 30 s, 72°C 1 min) × 26 cycles; 7S RNA (94°C 30 s, 52°C 15 s, 72°C 15 s) × 28 cycles. The Nep amplification products were fractionated on 1% (w/v) agarose gels, transferred to Nylon membranes Hybond-N+ Amersham Biosciences (Little Chalfont, Buckinghamshire, UK) and hybridized to Nep ^{32}P-labelled PCR-amplified probe in Church solution (0·5 mol l^{-1} $NaH_2PO_4.H_2O$, 0·5 mol l^{-1} Na_2HPO_4, 7% w/v SDS, 10 mmol l^{-1} EDTA) at 65°C for 16 h. The membranes were extensively washed at 65°C for 20 min with decreasing SSC concentrations, exposed to an imaging plate and analysed by the bioimaging analyser Storm 840 Amersham Biosciences (Sunnyvale, USA) and autoradiography. The amplification products of the 7S RNA were electrophoresed, stained with SYBR Gold staining (Invitrogen) and analysed by the bioimaging analyser Storm 840. Densitometry analyses of the RT-PCR images were performed using ImageQuant software.

Isolation of stimulatory 'factors'

CM and sterile medium were extracted three times with ethyl acetate (EA) 1 : 3 (v/v). The extracts were pooled and concentrated to 0·1 ml under reduced pressure on a rotary evaporator, poured into sterile flasks and dried under N_2. One volume of a culture of *Nab. magadii* ($OD_{600} \sim 0{\cdot}3$) was applied in each flask, and the cultures were incubated at 37°C with agitation. Samples were withdrawn throughout the growth curve and centrifuged at 12 000 ***g*** for 10 min. at 4°C. Cell-free media was used for the determination of protease activity.

Determination of protease activity

Protease activity was measured as previously described using azocasein as substrate (Giménez *et al.* 2000). One unit of protease activity was defined as the amount of enzyme that produced an increase of 1 in A_{335} per hour under the assay conditions.

Bioinformatic analysis

The NCBI protein database was searched for the bacterial quorum sensing (QS) proteins LuxI, LuxR, Pfs, LuxS, AinS, LuxM and HdsT (Fuqua and Greenberg 2002), and the relevant matches were used to build a phylogenetic tree using CLUSTALX (Thompson *et al.* 1997). One protein from each branch was selected and used as query in a BLAST search (http://www.ncbi.nlm.nih.gov) against the *Nab. magadii* ATCC 43099 complete genome (Genbank: ACIT00000000). ABC transporters and sensor proteins involved in QS were searched using the specific COG sequences (2274 and 2972, respectively) and their Peptidase C39B domain as query.

Results

Expression of *nep* is growth phase-dependent in *Nab. magadii*

Our previous work (Giménez *et al.* 2000) has shown that Nep activity accumulated in *Nab. magadii* cultures as the cells entered the stationary phase. To better understand this increase in protease activity, *nep* gene expression was examined at the mRNA, protein and activity levels along the growth curve. In addition, the effect of stationary phase CM on Nep biosynthesis was investigated. *Nab. magadii* cells were grown to mid-exponential phase (OD_{600} 0·3) and incubated in presence of CM or without additions (NA) until the cultures reached the stationary phase (Fig. 1a). Cell growth was similar in both cultures (g = 5 h), and the stationary phase was attained beyond 30 h of inoculation.

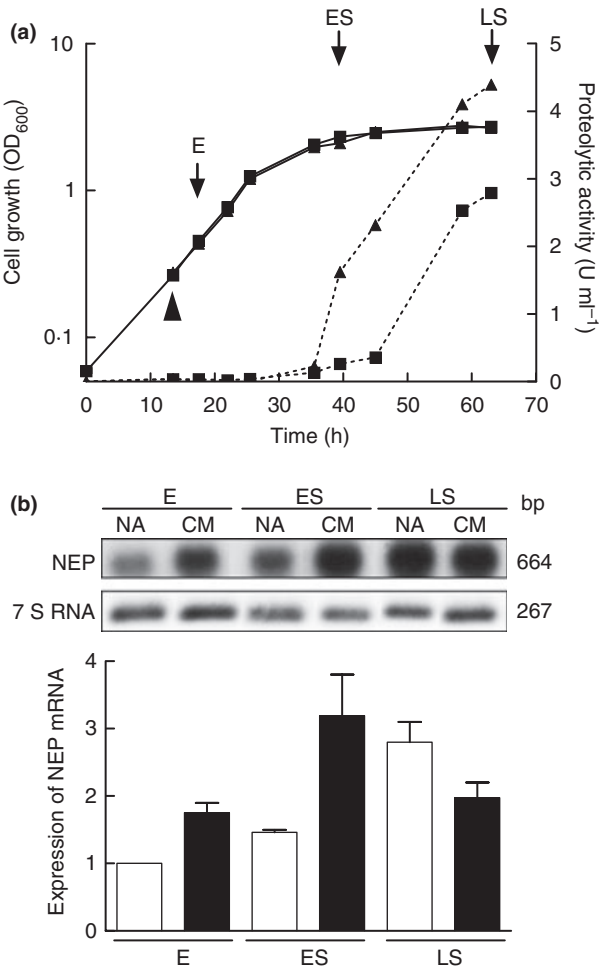

Figure 1 Growth phase and CM-dependent expression of *nep* gene. (a) Determination of cell growth (OD_{600}) (—) and extracellular azocaseinolytic activity (- - -); ■ NA, ▲ Conditioned media (CM). Protease activity was measured at 45°C for 16 h. The arrowhead indicates addition of CM and the arrows the time points that samples were withdrawn for RT-PCR analysis. Data are representative of at least five independent experiments. One such experiment is shown as supplementary material (Fig. S1a). (b). RT-PCR analysis of Nep and 7S RNA transcripts. The standard deviation of duplicate RT-PCR is shown. NA, empty bars; CM, filled bars. E, exponential; ES, early stationary; LS, late stationary growth phases; CM, cells incubated with CM; NA, no additions. Data are representative of two independent experiments.

Protease activity was detected in the culture medium beyond 45 h in the control; however, it was observed after 35 h in the culture incubated with CM. In both cultures, the activity accumulated in the LS phase reaching 2·8 and 4·5 U ml^{-1} in control and CM-treated cultures, respectively. Relative amounts of Nep mRNA were estimated by semi-quantitative RT-PCR analysis. An internal fragment of the expected size (664 bp) was amplified from Nep transcript in exponentially growing cells whose abundance

increased as the cells entered the stationary phase (Fig. 1b) in parallel with increased amounts of Nep in the culture medium, as evidenced by Western blotting with anti-Nep antibodies (not shown), and the concomitant accumulation of extracellular protease activity (Fig. 1a). In an independent experiment, higher levels of Nep mRNA as the culture aged were estimated by dot blotting. To normalize the amounts of Nep mRNA, a fragment of *Nab. magadii* 16S rRNA transcript was amplified from the cDNA template. A decrease (about 2-fold) in the 16S RNA hybridization signal was observed in stationary phase cells suggesting that the expression of the 16S rRNA gene was negatively affected by the growth stage in *Nab. magadii.* Thus, we searched for a constitutively expressed gene in *Nab. magadii.* The 7S RNA was used to normalize the expression of halocin C8 in the haloarchaeal strain AS7092 in Northern blots (Sun *et al.* 2005). Primers for the *Nab. magadii* 7S RNA were synthesized based on the genomic sequences of *Nab. magadii* ATCC 43099 and used in RT-PCR to determine the amount of this transcript along the growth curve. The relative cellular concentration of the 7S RNA was similar in *Nab. magadii* cells at different growth stages (Fig. 1b); therefore, *nep* gene expression was estimated as the ratio of Nep mRNA amount divided by that of the constitutively expressed 7S RNA transcript. Increments of 1·5- and 3-fold in Nep mRNA were estimated in early stationary (ES) and LS cells relative to E cells. Interestingly, 2-fold increase in Nep mRNA compared to controls was observed in CM-treated E and ES cells (Fig. 1b) (corresponding to 4 and 26 h after addition of CM), consistent with the anticipated accumulation of protease activity in the extracellular medium (Fig. 1a). Although significant amounts of Nep mRNA were present in E cells (mainly in CM treated), no extracellular protease activity was detected suggesting that Nep may not be efficiently translated and/or transported in E cells or, alternatively, the sensitivity of the protease assay did not allow its detection.

Altogether, these results show that accumulation of protease activity in the extracellular medium as the culture ages is due to increased levels of Nep mRNA and protein, demonstrating that *nep* gene expression is up-regulated during the transition to the stationary phase. On the other hand, Nep synthesis was attained at earlier stages of growth when the cells were incubated with stationary phase CM (Fig. 1) showing a stimulatory effect of CM on Nep production. This effect may be attributed to removal of an inhibitor of protease synthesis or to the presence of a stimulatory 'factor'.

Extracellular 'factors' induce *nep* expression

To better understand the effect of CM on Nep synthesis, attempts were made to isolate a potential stimulatory 'factor'. Based on previous results, which suggested the presence of autoinducer molecules of the homoserine lactone-type in the haloalkaliphilic archaeon *Natronococcus occultus* (Paggi *et al.* 2003), we sought for the presence of QS autoinducers. CM and sterile medium were extracted with EA. These fractions were added to low-density cultures of *Nab. magadii* to induce protease activity. Addition of CM-EA extract did not affect cell growth (g = 5 h); however, it induced early accumulation of proteolytic activity relative to the control culture (Fig. 2).

In addition, CM and sterile medium were fractionated by Sep-pack C18 cartridges (Waters Inc., USA) and eluted with a step-gradient of methanol:water. *Nab. magadii* cells incubated in presence of the 30% methanol CM fraction produced higher levels of Nep activity (4-fold) relative to the control.

Altogether these results suggest that *Nab. magadii* cells secrete hydrophobic molecules that elicit Nep activity. These findings are promising; however, the lack of reproducibility in replica experiments as well as the presence of contaminants did not allow the identification of these molecules.

Bioinformatic analysis of QS systems in *Nab. magadii*

The availability of the *Nab. magadii* ATCC 43099 genome sequences allowed the *in silico* survey for putative genes homologous to bacterial QS components. The result of such analysis did not allow the identification of proteins similar to those involved in bacterial QS in the archaeon *Nab. magadii.*

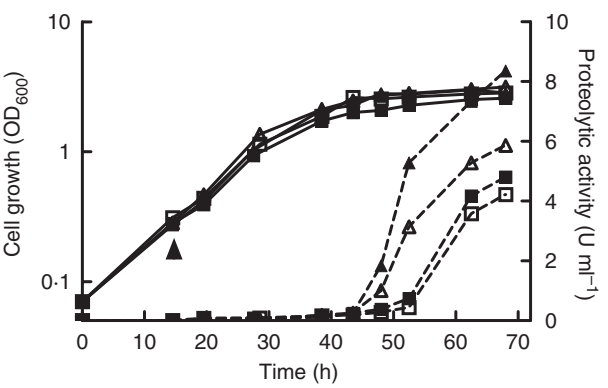

Figure 2 Isolation of 'factors' that stimulate Nep biosynthesis. Conditioned media (CM) and sterile medium were extracted with EA. The extracts were evaporated and added to low-density cultures of *Natrialba magadii* (OD_{600} 0·3) (arrowhead). Samples were withdrawn for determination of cell growth (OD_{600}) (—) and extracellular azocaseinolytic activity (- - -); ■ sterile medium, ▲ CM. Protease activity was measured at 45°C for 5 h. Data points of two independent experiments are shown (empty and filled symbols).

Discussion

This study shows that *nep* gene expression is up-regulated during the transition to the stationary phase in *Nab. magadii* (Fig. 1). This finding supports our previous observations, which showed that carbon and nitrogen starvation did not induce protease accumulation, suggesting that *de novo* synthesis of Nep was required (D'Alessandro *et al.* 2007). From a physiological view point, a high concentration of Nep in the extracellular medium at elevated cell densities would allow a more efficient scavenging of protein/peptide substrates in the natural environment of this microbe.

In addition, a stimulatory effect on protease biosynthesis was attained in exponentially growing cells challenged with stationary phase-CM suggesting that either an 'inhibitor' of Nep synthesis may have been consumed in the CM and/or 'factors' which induce protease synthesis accumulated in the extracellular medium of the growing cells. These 'factors' may be metabolic end-products and/or regulatory molecules secreted by *Nab. magadii* cells.

QS is a cell-to-cell signalling mechanism that allows bacteria to monitor their population density by secreting and sensing small molecules (Reading and Sperandio 2006). Bacteria use QS for the regulation of many biological functions, including the synthesis of hydrolytic enzymes. This cell-to-cell communication strategy is widespread in pathogenic and symbiotic bacteria, but there exists limited information on the occurrence of QS-like signalling in extremophiles, including archaea (Farah *et al.* 2005; Johnson *et al.* 2005; Llamas *et al.* 2005; Rivas *et al.* 2005, 2007; Sewald *et al.* 2007; Nichols *et al.* 2009). It has been suggested that QS controls entry into the stationary phase, nutrient limitation and stress response in bacteria (Lazazzera 2000). Taking into account that QS autoinducers are secreted to the extracellular medium, we examined the possibility that the extracellular 'factors' that stimulated Nep biosynthesis may be QS-like autoinducers. We attempted to isolate these molecules using two procedures that have been used for the isolation of QS autoinducers in bacteria. In these experiments (Fig. 2), we obtained preliminary evidence that CM contains molecules of hydrophobic nature which stimulate Nep production. Unfortunately, the results were not always reproducible preventing the identification of these molecules. This fact may be attributed to the harsh conditions used during purification of these 'factors' from the haloalkaliphilic medium (3·5 mol l^{-1} NaCl and pH 10) and/or an 'unusual' chemical nature of these molecules, which may have affected their solubility in the solvents applied. The purification strategy is currently being optimized to improve the isolation of the putative 'factors' produced by *Nab. magadii* for further identification and characterization.

The *in silico* analysis of the complete genomic sequences of *Nab. magadii* ATCC 43099 did not show the existence of candidate protein–coding genes homologous to bacterial QS components in this unusual microbe. However, the possibility that archaea, including *Nab. magadii*, might possess nonconventional pathways for the production of novel autoinducer molecules cannot be ruled out.

Although so far the molecular mechanism involved in the regulation of Nep synthesis has not been addressed, this study shows that 'factors' (metabolite, regulatory molecule) occurring in spent medium of *Nab. magadii* are responsible, at least in part, for the enhanced synthesis of Nep at the onset of the stationary phase. Nep biosynthesis appears to be an excellent model to study gene expression and cell signalling in alkaliphilic haloarchaea, a field that has not been investigated.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Growth phase and CM-dependent expression of *nep* gene.

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